SKELETON FORMATION OF SEA URCHIN LARVAE. I. EFFECT OF CA CONCENTRATION OF THE MEDIUM [1]

KAYO OKAZAKI

Department of Biology, Tokyo Metropolitan University, Setagaya-ku, Tokyo and Misaki Marine Biological Station, Misaki, Miura-City, Japan

Although many reports are available concerning the morphological development of the sea urchin skeleton (v. Ubisch, 1913; MacBride, 1914; Mortensen, 1921, 1931; Goldon, 1926a, 1926b, 1928; Onoda, 1931, etc.), the underlying mechanism of its formation is an aspect which has not been covered by previous workers.

In marine molluscs, on the other hand, it is known that the calcium utilized in the formation of the shell is derived in part from the sea water in which the animals live. Galtsoff (1934) reported that the amount of calcium deposited to form the oyster shell was many times greater than that which could be stored in the tissues. Orton (1925) noticed in the oyster that increase in the size of the shell took place even in the absence of food. Fox and Coe (1943), in their studies on *Mytilus*, showed that the amount of calcium obtained from organic food alone was not sufficient to account for the amount deposited in the shell. In recent studies, it has been proved that an isolated "mantle-shell preparation" is able to deposit calcium from the sea water onto the shell (Hirata, 1953; Jodrey, 1953). Bevelander and Benzer (1948) have shown that there is a corresponding reduction in the calcium of the regenerating molluscan shell (*Pedalion alatum*) when the calcium content of the sea water is reduced. Further, Bevelander (1952) agrees with Robertson (1941) in the conclusion reached in Robertson's comprehensive review dealing with calcification in molluscs, that molluscs can utilize calcium in the ionic form. This conclusion is further corroborated by Rao and Goldberg (1954), who cultured *Mytilus* in filtered sea water.

In sea urchin larvae, a direct dependence on sea water for the salts or ions used for the larval skeleton is shown by the following facts: the beginning of spicule formation precedes the completion of gastrulation, hence takes place before feeding starts; spicules continue to grow in cultures which are without food; exogastrulae having no functional digestive tract are able to form typical skeletons like those of normal plutei (Okazaki, 1956). In earlier studies, it has been stated that spicules do not develop in sea water in which the calcium is seriously reduced (Pouchet and Chabry, 1889) and also that the absence of magnesium and sulphate ions arrests the spicule formation of sea urchin larvae (Herbst, 1904). However, hardly any details about skeleton formation have been clarified so far.

In the present paper, the effects of the Ca concentration of the culture medium on skeletal development will be reported as a preliminary to the study of the mechanism of skeleton formation.

[1] This research was supported in part by the Ministry of Education Research Expenditure.

Materials and Methods

The larvae of *Hemicentrotus pulcherrimus* were principally used, because the form of the skeleton of this species is the simplest to be found among the sea urchin larvae available at Misaki. As supplemental materials, *Pseudocentrotus depressus*, *Clypeaster japonicus* and *Mespilia globulus* were used.

Ca-low media were prepared by mixing Ca-free sea water [2] and natural sea water in 9 : 1, 8 : 2, 6 : 4, 4 : 6 and 2 : 8 ratios. As Ca-high media, 0.36 *M* $CaCl_2$ was mixed with natural sea water in 11 : 629, 11 : 309, 11 : 149, 11 : 69 and 11 : 29 ratios. The resultant Ca concentrations of the whole series of mixtures correspond to about 0.1 ×, 0.2 ×, 0.4 ×, 0.6 ×, 0.8 ×, 1.5 ×, 2.0 ×, 3.5 ×, 6.0 × and 10.0 × the calcium content of natural sea water. For convenience, these media will be referred to as 0.1 Ca, 0.2 Ca, 0.4 Ca . . . , respectively.

Larvae were cultured in Syracuse watch glasses containing about 15 cc. of medium. In these cultures, the larvae must be placed in a single layer, so as to minimize variations in the rate of development which would lead to individual differences in skeletal size. As long as the number of larvae in a container is kept below such a level, further reduction in the larval number per container does not affect the skeleton formation. This latter situation indicates that it is the concentration of calcium and not the absolute quantity of calcium available per larva which should be taken into consideration.

In all the experiments reported in this paper, the larvae were cultured in the absence of food.

Experimental Results

Before describing the effects of media with modified Ca concentrations on skeleton formation, it seems appropriate to mention their effects on the general developmental process of sea urchin larvae. These are summarized in the following five points.

(1) *Time of hatching.* In Ca-low media, no retardation of development was obvious in reference to the hatching time, while in Ca-high media, the time of hatching was more or less retarded. However, this retardation of hatching time in Ca-high media is not simply due to a generally delayed rate of development, judging from the time of mesenchyme formation and gastrular invagination, as described below.

(2) *Time of beginning of mesenchyme immigration and gastrular invagination.* With reference to the time of the onset of these activities, larvae in 0.1–3.5 Ca did not show any difference from the control, except occasional early beginning in 0.1 and 0.2 Ca. In 6.0 and 10.0 Ca larvae, however, both activities were retarded in comparison with the controls.

(3) *Gastrulation.* In 0.2–6.0 Ca, gastrulation proceeded in an almost normal manner. Larvae cultured in extreme concentrations (0.1 and 10.0 Ca) frequently developed into exogastrulae, as described in previous papers (Dan and Okazaki, 1956; Okazaki, 1956).

(4) *Size of the blastocoel.* In Ca-low media, the size of the larval blastocoel

[2] NaCl 26.5 g., KCl 0.7 g., $MgSO_4 \cdot 7H_2O$ 11.9 g., $NaHCO_3$ 0.5 g. plus distilled water to 1000 cc.

became larger than that of the control larvae. This was so particularly in 0.8 and 0.6 Ca. In Ca-high media, on the other hand, the blastocoels became smaller in proportion to the concentration of Ca present, in the same manner as was reported by Dan (1952) in this species.

(5) *Formation of coelom.* In the extreme concentrations, the secondary mesenchyme cells failed to form a typical coelom. They either formed a single vesicle on the tip of the archenteron or dispersed without coordination.

From the foregoing facts, it can be said that the concentration range of Ca which allows essentially normal development is from 0.2 to 3.5 times that of sea water.

I. Limiting concentration of calcium necessary for skeleton formation

In larvae which were cultured in even as low a concentration as 0.1 Ca from the two- or four-cell stage, doubly refractive skeletons were formed, although their form was atypical and the time of their formation was much delayed, as will be described below. If larvae reared in sea water were transferred to Ca-low sea

TABLE I

Comparison of the time of the beginning of the skeleton formation in various Ca concentrations. Numbers indicate the ratios of hours required between insemination and skeleton formation in experimental media over the control. Material: Hemicentrotus pulcherrimus

Stage of transfer \ Ca concentration	0.1	0.2	0.4	0.6	0.8	S.W.	1.5	2.0	3.5	6.0	10.0
4-cell						1.00	0.99	1.00	1.00	1.04	1.08
32-cell	2.22	1.69	1.31	1.03	1.00	1.00	1.00	1.00	1.00	1.05	1.07
Morula	2.80	1.87	1.17	1.05	1.01	1.00	1.00	0.99	1.00	1.05	1.07
Blastula before hatching	2.65	1.71	1.15	1.02	1.00	1.00	0.99	0.99	1.00	1.04	1.13
Swimming blastula with no mesenchyme cells	2.20	1.69	1.29	1.03	1.00	1.00					

water after the formation of tri-radiate spicules, skeletal growth continued for a short time even in 0.05 Ca, in which the general development was ultimately impaired. These facts show that the Ca threshold for skeleton formation is lower than that for harmonic development of the whole larva. Such being the case, the minimal calcium concentration required for skeleton formation itself is impossible to decide.

Bevelander and Benzer (1948), in their paper dealing with shell regeneration in young specimens of *Pedalion,* reported that when an operated animal was placed in sea water containing $\frac{1}{8}$ of the normal calcium content, the organic matrix was completely devoid of calcium, although the matrix was formed in a normal manner. For sea urchin larvae, however, a medium having such an effect cannot be prepared by calcium reduction alone.

It is frequently the case that in the larvae of *Clypeaster japonicus* and *Mespilia globulus* cultured in 0.1 Ca, skeletons as such failed to appear; instead, minute doubly refractive granules were formed in the mesenchyme cells.

II. Time of the beginning of skeleton formation

Larvae fertilized and cultured in sea water were transferred to media of various calcium concentrations at various stages before the appearance of the mesenchyme

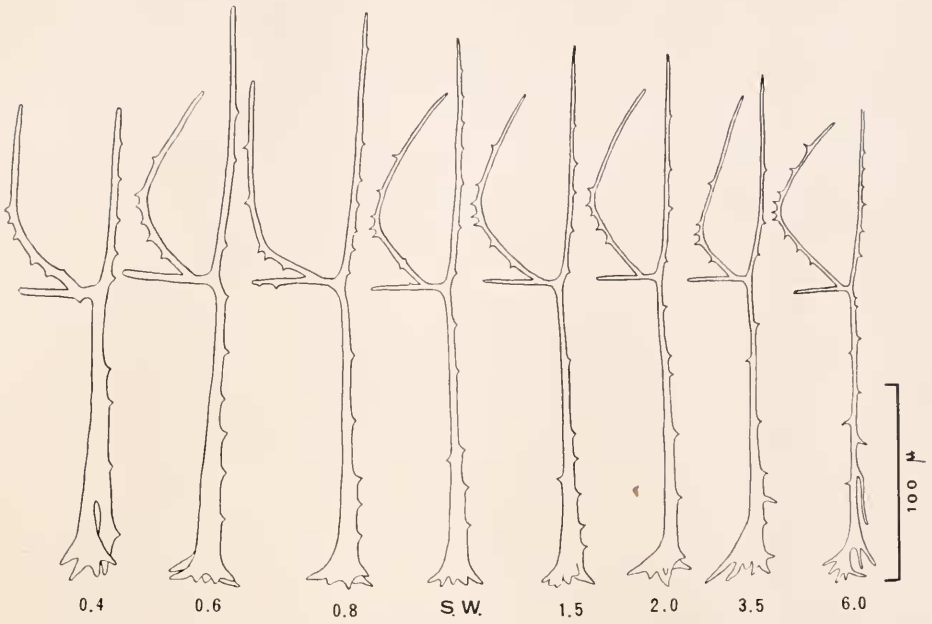

FIGURE 1. Skeletons of *H. pulcherrimus* larvae reared in media with varied Ca concentrations from 0.4 to 6.0 times that of sea water.

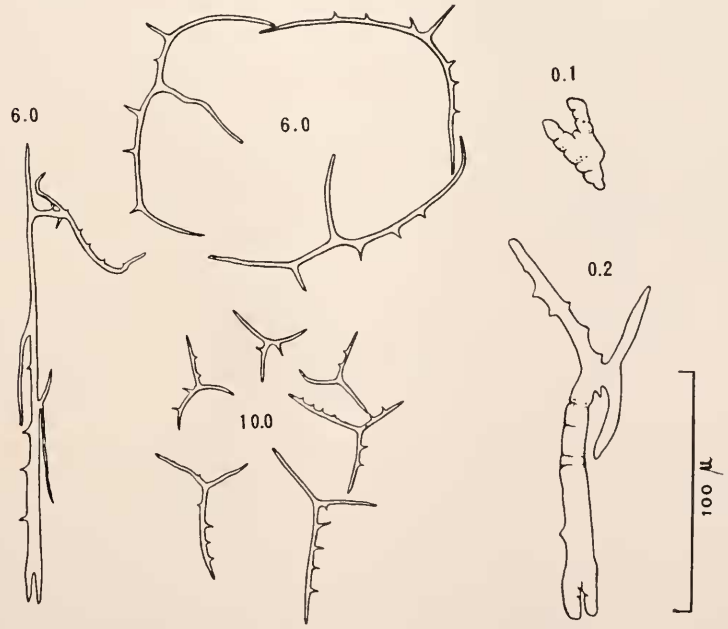

FIGURE 2. Skeletons of *H. pulcherrimus* larvae in the extreme Ca concentrations (0.1, 0.2, 6.0 and 10.0 × normal sea water.) Larvae were transferred to such media before skeleton formation.

cells, and the times of the beginning of skeleton formation were compared. As the initial stage of skeleton formation, the time was recorded when doubly refractive granules were recognized by a polarization microscope in 50% of the larvae in a given culture. The results are summarized in Table I.

As far as pre-mesenchyme blastulae were concerned, the stage at which the larvae were transferred did not affect the results. Generally speaking, in Ca-low media, the time of the beginning of skeleton formation became later in proportion

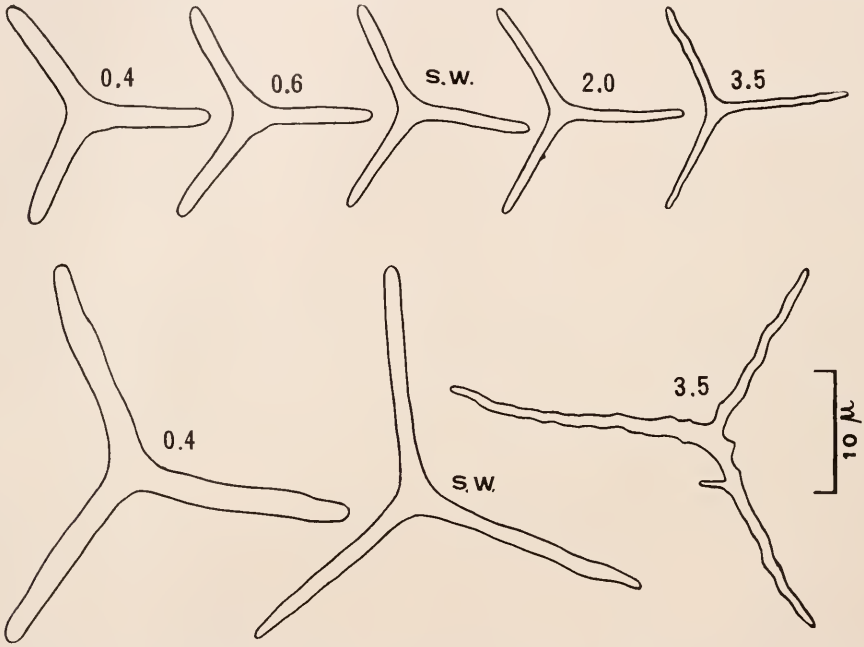

FIGURE 3. Camera lucida drawings of spicules in media of various Ca concentrations at two stages of skeletal development. Note correlation between Ca decrease and thickening of spicules. Larvae transferred to experimental media from sea water at gastrula stage with rudimental spicule granules.

to the dilution of Ca, while the increase in Ca content had only a slight effect in this sense. In considering the delay in skeleton formation at extreme concentrations, the retardation of the developmental rate must also be taken into account.

III. Form of the skeleton in calcium-low and -high media

When larvae were cultured in media with various Ca concentrations from a stage before the beginning of skeleton formation, the forms of the skeletons of these larvae at "the final stage" [3] showed characteristic features according to the calcium content, as is illustrated in Figures 1 and 2.

[3] By "the final stage" is meant the stage in which the larval skeleton reaches its maximum of development in foodless culture. Control larvae at this stage have four long arms, a func-

On the whole, in the range of 0.4–6.0 Ca, the skeletal forms of the experimental plutei were roughly similar to those of the controls (Fig. 1). If they are compared in detail, however, a slight shift can be recognized in the thickness and length of the skeleton, and in the shape and number of hooks, paralleling the concentration shifts in calcium. The thickening of the skeleton was inhibited in proportion to the increase in the Ca content of the medium; this was noticeable even as early as the tri-radiate spicule stage, as is indicated in Figure 3. The elongation of the skeleton

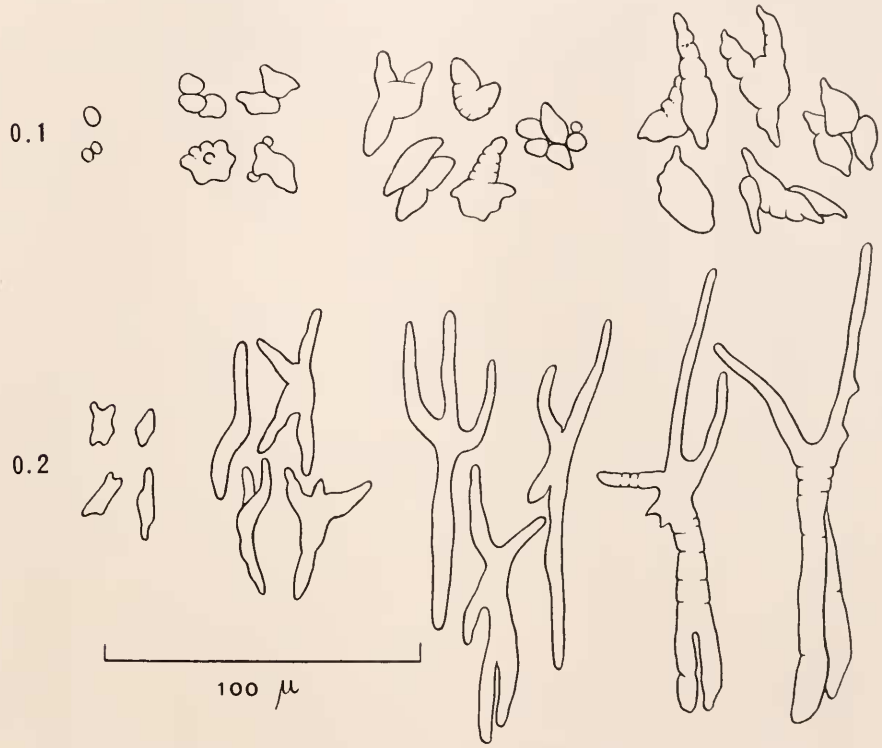

FIGURE 4. Process of skeletal growth in *H. pulcherrimus* larvae in Ca-low media (0.1, 0.2 × Ca).

was inhibited in proportion to the decrease in Ca concentration within the range 0.1–0.4 Ca (see Figs. 1, 2). There was a corresponding reduction in the number and the sharpness of the hooks, if the calcium content of the medium was reduced, and finally in 0.1 Ca, the hooks were missing.

At extreme concentrations outside the above range, there occur distinct differ-

tional digestive tract and a pair of coeloms. Since, however, the destined length of life of the experimentals turns out to be as long as that of the controls or even longer in some solutions, the data of the controls and experimentals are roughly comparable. When experimental larvae died before the end of the experiment, they were omitted from the data.

ences, with respect to the form and developmental process, between the skeletons of controls and of experimentals, as will be described below.

Skeletons in media extremely low in calcium. As was mentioned above, doubly refractive skeletons could be formed in larvae reared in such Ca-low media as 0.1 or 0.2 Ca from a stage before skeleton formation. In such media, these doubly refractive skeletal rudiments never developed into the tri-radiate form found in the controls. However, the rudiments continued to grow in size as irregular masses (Fig. 4). As a natural consequence, the skeletons at the final stage of such Ca-low larvae were extremely atypical.

On the other hand, if the larvae were transferred to 0.1 or 0.2 Ca after the formation of tri-radiate spicules, new skeletal development in the Ca-low media was

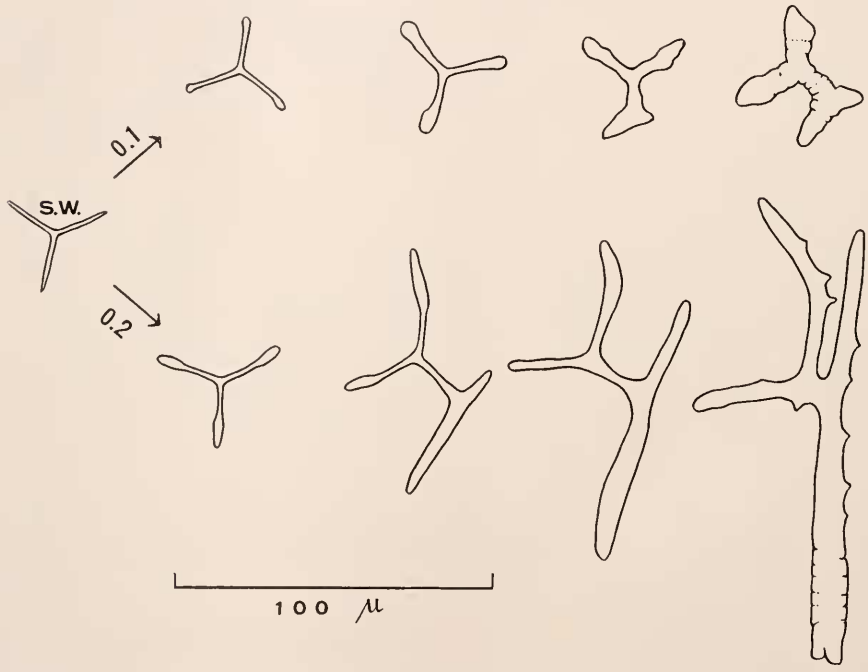

FIGURE 5. Modification of shape of tri-radiate spicules on transfer to Ca-low media (0.1 and 0.2 Ca).

recognized as thickenings at each of the three spicule tips, globular in 0.1 Ca and ovoid in 0.2 Ca. (This fact may indicate that in the normal process of skeleton formation, Ca deposition is more vigorous at the tips than at the other points of the skeleton.) Subsequently, all parts of the spicules gradually thickened until skeletons at the final stage resembled typical pluteus skeleton, although they were much dwarfed (Fig. 5).

If the larvae were transferred to Ca-low media after the pluteus stage, the thickening of the skeleton was found first at all tips and hooks of the skeletons (Fig. 6). In all cases in the two media lowest in Ca, it was a conspicuous feature of skeletal growth that the proportion of length to thickness was very small, par-

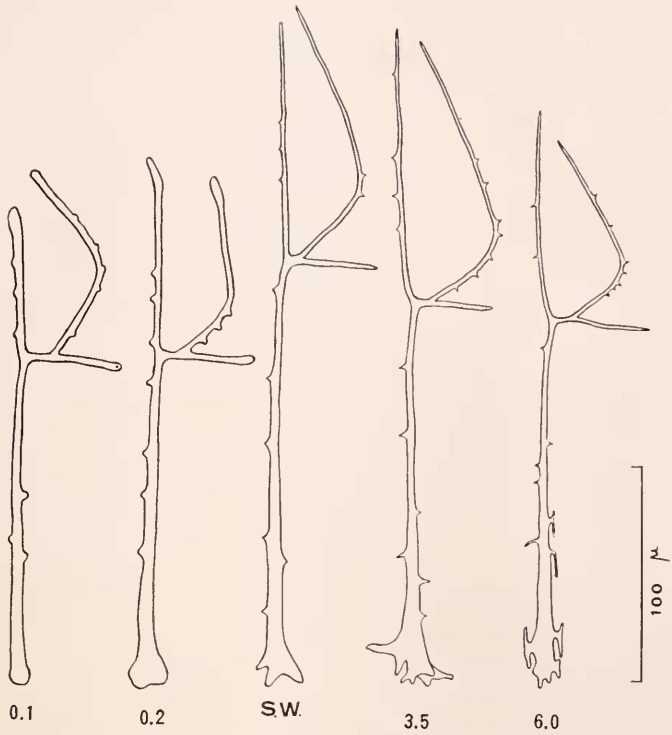

FIGURE 6. Effect of late transfer of *H. pulcherrimus* plutei to experimental media. Note effects of Ca change on form of hooks and skeletal tips.

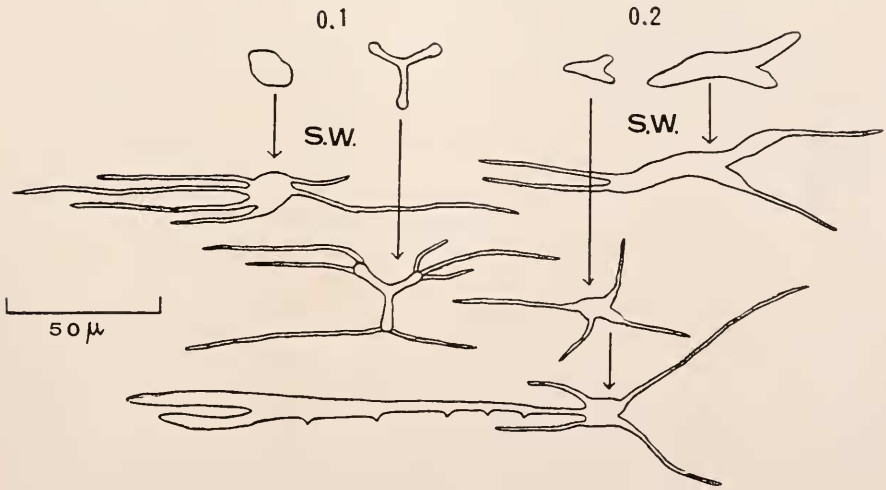

FIGURE 7. Skeletons of *H. pulcherrimus* larvae transferred back to sea water from Ca-low media (0.1 or 0.2 Ca), showing terminal elongation of slender skeletal rods.

ticularly in 0.1 Ca, and the surface of the skeleton became uneven as it thickened, as is shown in Figures 2, 4 and 5.

As a converse experiment to the above cases, if larvae which had been in 0.1 or 0.2 Ca were returned to sea water during the course of skeleton formation, several spindly processes invariably grew out from the edge of the massive skeleton, without regard to the stage of transfer (Fig. 7). In this case, if the larvae were transferred to 0.4, 0.8 or 1.0 Ca (sea water), the shape of the parts newly formed on the old one became longer and thinner in proportion to the new concentration of Ca.

Skeletons in extremely high calcium media. If the larvae were cultured in 10.0 Ca from a stage before skeleton formation, several spicule centers were formed, each of which developed into a tri-radiate spicule. These rarely attained a sufficient size to serve as functional pluteus skeletal parts (Fig. 2). Such abnormality was sometimes obtained in 6.0 Ca larvae. However, if sea water larvae were transferred to 6.0 or 10.0 Ca after the formation of tri-radiate spicules, excess centers of the spicules did not form and new skeletal development was continued to form the typical pluteus skeleton on the basis of the old spicules. In either case, the usual thickening failed to take place in such skeletons and there was also some reduction in elongation.

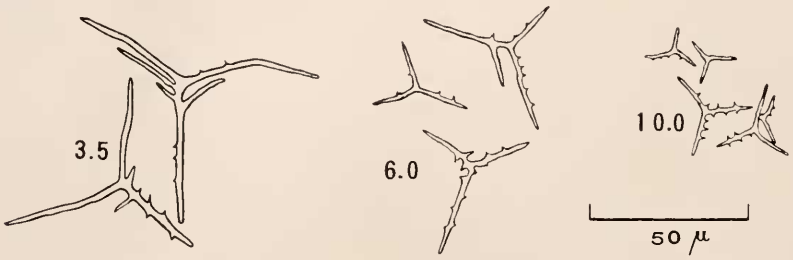

FIGURE 8. Spicules of *Hemicentrotus* gastrulae having precocious hooks in Ca-high media.

In 6.0 or 10.0 Ca, the side hooks of the skeleton which normally appear at the pluteus stage were formed prematurely at the gastrula stage, *i.e.*, at the tri-radiate spicule stage (Fig. 8). Such precocious development of the side hooks was also frequently observed in 3.5 Ca. If the transfer to Ca-high media was done after the pluteus stage, the shape of the hooks became sharper and longer as the Ca content of the medium was increased (Fig. 6).

IV. The effect of Ca concentration on apparent increase in length of the skeleton

Putting aside the aberrant skeletons formed in the two extremes of the Ca range, a comparison of the skeletal growth by microscopical measurement was made within the range of 0.4–3.5 Ca. Since the skeletal growth of individual larvae was very variable even among control larvae derived from the same batch, and further, since these individual differences became increasingly greater with the increasing divergence of the Ca concentration from that of sea water, particularly on the dilute side, the data given are averages of specimens showing good development (see Glaser, 1950).

To obtain uniform initial length of the spicules, the larvae were allowed to de-

velop in sea water until the formation of the tri-radiate spicules had taken place, before they were transferred into media of the above range of calcium, and the lengths of the spicules in the various media were compared at intervals thereafter. In spite of variation in the details of the apparent skeletal growth, the general trend showed that a high concentration of calcium caused better skeletal growth

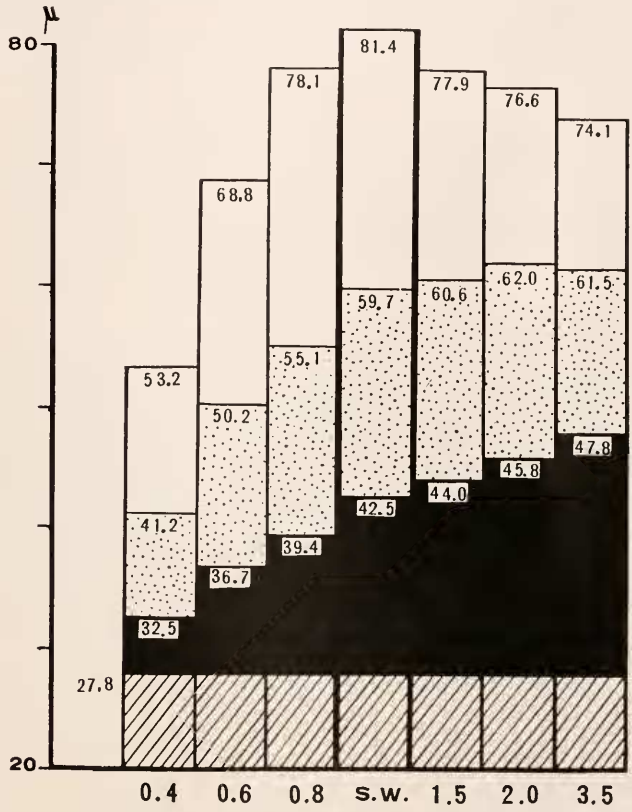

FIGURE 9. Apparent skeletal growth following transfer from sea water to media with various Ca concentrations. Material: *Hemicentrotus pulcherrimus* gastrulae. Ordinate: actual lengths of spicules calculated from camera lucida drawings. Abscissa: Ca concentrations of media. Columns with shading indicate initial length of spicules at transfer. Black, dotted and white columns indicate increase in skeletal length during each two hours after transfer at 16.5° C.

than sea water for a short time following transfer, but became harmful later on, while low calcium was relatively harmless.

The data of one experiment presented in Figure 9 illustrate this tendency. In this case, the length of the skeleton reached during the first two-hour period after transfer increased corresponding to the Ca concentration.[4] For the second

[4] Maximum increase in skeletal length during the first period was found sometimes in 1.5 Ca or in 2.0 Ca or in 3.5 Ca. In all cases, however, the skeletons formed in Ca-high media were longer than those in sea water.

two-hour period, however, the growth rate of the skeleton in the Ca-high media came down to approximately the same level as that of sea water, and for the third two-hour period, the increase in skeletal length in Ca-high media became inferior to that in sea water. On the other hand, the growth rate in Ca-low media decreased in proportion to the decrease in Ca concentration at first, but improved later.[5]

If the total lengths of post-oral rod + body rod at the final stage in various media were compared, the skeletons in 0.6 or 0.8 Ca were usually longer and in Ca-high media were shorter than the skeletons in sea water.

V. Spatial relations between ectoderm and skeleton

At the time when tri-radiate spicules were formed in sea water, spicules had not yet been formed in 0.1 or 0.2 Ca, but the ectoderm at the position of the presumptive post-oral arms was wrinkled (Fig. 10). If such larvae were transferred to sea water, the wrinkled ectoderm gradually expanded and formed the arm, accompanied by development of the spicules. However, if the larvae were kept in 0.1 Ca, the

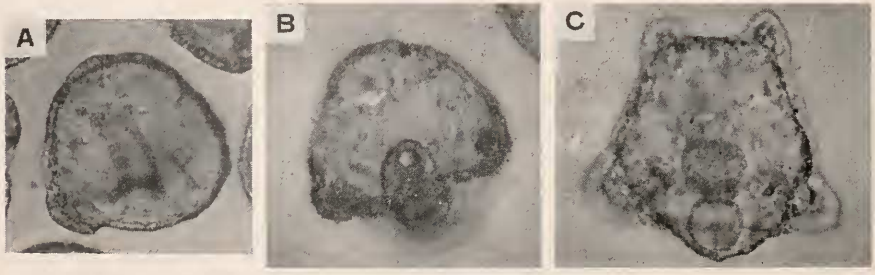

FIGURE 10. *H. pulcherrimus* larvae cultured in Ca-low medium. A, B: 0.1 Ca gastrulae, showing wrinkles of the ectoderm at position of presumptive post-oral arms. A: side view. B: vegetal view. C: 0.2 Ca pluteus, showing post-oral and antero-lateral lobes without rods.

wrinkles became deeper as development advanced, and frequently the wrinkled ectoderm fell off from the body as small vesicles, although in 0.2 Ca, the spicules eventually developed and short arms grew out.

At the stage in which the antero-lateral arms developed in sea water larvae, the ectoderm covering the area of the presumptive antero-lateral arms of 0.1 or 0.2 Ca larvae formed a process which could virtually be considered as an antero-lateral lobe, in spite of the fact that the antero-lateral rod was experimentally suppressed in these larvae (Fig. 10). On transfer back to sea water, this lobe developed into an antero-lateral arm supported by an attenuated skeleton. If several of these slender skeletal extensions grew simultaneously from the central mass, a single branch which elongated in the direction of the lobe acted as a substitute for the antero-lateral rod, but arms never grew out where other branches reached the ectoderm. On continuous culture in 0.1 or 0.2 Ca, the antero-lateral lobe gradually disappeared instead of being cut off from the body. Rulon (1941) reported that *Dendraster excentricus* embryos treated with NaSCN developed into larvae having the general

[5] The growth of the skeleton during the third period was frequently better in 0.8 Ca than in sea water.

shape of plutei in spite of the lack of the mesenchyme cells and a gut. The Ca-low larva reported here (Fig. 10) is very similar in shape to this larva of Rulon's (Fig 12 in his paper).

From the above fact, it can be said that the ectoderm which is to develop into the arm is able to self-differentiate, but for a normal arm to result, a spatial relation between the ectoderm and the skeleton is necessary. This has already been pointed out by Jenkinson (1911), MacBride (1914), Runnström (1931), v. Ubisch (1933) and Hörstadius (1935).

Discussion

As was described in Section II, the higher the concentration of calcium ions, the earlier is the time at which skeleton formation begins. From this fact it is to be expected that the skeletal growth in Ca-high media will be faster than in normal or Ca-low sea water. Such a situation, however, was found to obtain only for a short while after the calcium change (first two hours in Fig. 9). After this period, the skeletal growth was rather accelerated in such low concentrations of calcium as 0.6 or 0.8 Ca, and inhibited by high concentrations. It is still impossible to consider the above facts in terms of the rate of Ca deposition, since it is doubtful whether the apparent size variation of the skeleton is proportional to the quantity of deposited calcium. Abe and co-workers (1954) have reported on calcium deposition by adult oysters which were kept in media of different concentrations of radio-active calcium; if twice as much calcium was provided calcium deposition was slightly increased, while in the medium of half calcium, the deposition of calcium was reduced to nearly one-tenth that found in sea water.

There is another situation to be considered in sea urchin larvae. The spicules of these larvae always grow along a row of primary mesenchyme cells. This fact suggests that the direction of spicule elongation is decided by the mode of arrangement of the mesenchyme cells, and the length of the skeleton is conditioned by the distance to which they extend. The primary mesenchyme cells are further apart in low calcium because of the larger blastocoelic volume, and are brought closer together in high calcium by the smaller size of the blastocoel. On such a basis, it becomes understandable why the apparent growth of the skeleton in 0.6 or 0.8 Ca was rather superior, and that in Ca-high media was inferior to the growth of the controls.

Woodland (1906) and Prenant (1926) suggested from observations of normal skeletal development that a sheath-like structure which is found along the row of primary mesenchyme cells may be a matrix for the skeleton. If this is true, it must be concluded that the apparent length of the skeleton is conditioned by the length of the skeletal matrix which, in turn, is proportional to the distance covered by the longitudinally arranged mesenchyme cells. And further, the tendency that the ratio of length to thickness of the skeleton becomes smaller as the amount of available calcium decreases will find its explanation in the deformation of the matrix resulting from the change in the calcium content of the medium. A detailed description of the skeletal matrix will be reported in the next paper.

The writer wishes to express her sincere thanks to Professor K. Dan and Dr. J. C. Dan for their kind criticism and valuable advice as well as for their assistance

in the preparation of the manuscript. The writer's thanks are also due to the director and staff of the Misaki Marine Biological Station for use of the Station's research facilities.

Summary

1. The threshold Ca concentration for the growth of the larval skeleton is lower than that which permits the normal development of sea urchin larvae.

2. The time of the beginning of skeleton formation is retarded by a decrease in Ca concentration from the normal level, while an increased Ca concentration has little effect in this respect.

3. Modification of the Ca concentration of the medium influences the form of the skeleton of sea urchin larvae.

a). In larvae transferred to very dilute calcium before the time of skeleton formation, a mass of unorganized skeletal substance develops.

b). In larvae put into very concentrated calcium from a similarly early stage, several tri-radiate spicules are formed in each larva, but these fail to make a typical skeleton.

c). If the transfer is deferred till the tri-radiate spicules are formed, new skeletal development in the experimental media is continued on the old spicules, and a pluteus skeleton which is similar to the typical one results, though it is much stunted in Ca-low, and slender in Ca-high media.

d). The proportion of the thickness to the length of the skeleton is inversely proportional to the Ca concentration. This is true regardless of the stage of transfer.

e). The elongation of the skeletal rods is inhibited at the extremes of the Ca-series, particularly on the dilute side.

f). The apparent increase in skeletal length is rather better in 0.6 or 0.8 Ca than in high-calcium, except that the contrary situation holds for a short time after transfer.

4. The ectoderm of the presumptive arm may differentiate without any skeleton. However, such differentiated ectoderm finally falls off from the body or disappears unless the supporting skeleton develops.

5. It is suggested that an organic matrix may be formed before deposition of the mineral components of the skeleton; that this matrix is deformed by changes in the calcium concentration; and that such deformation, in turn, influences the rate of growth and the form of the skeleton.

LITERATURE CITED

Abe, Y., Y. Kado, M. Shinagawa, J. Tanaka and S. Yajima, 1954. Measurements of biochemical deposition of calcium by oyster kept in sea water of three different calcium content labeled with Ca^{45}. *J. Sci. Hiroshima Univ. Series A*, **18**: 249–252.

Bevelander, G., 1952. Calcification in molluscs. III. Intake and deposition of Ca^{45} and P^{32} in relation to shell formation. *Biol. Bull.*, **102**: 9–15.

Bevelander, G., and P. Benzer, 1948. Calcification in marine molluscs. *Biol. Bull.*, **94**: 176–183.

Dan, K., 1952. Cyto-embryological studies of sea urchins. II. Blastula stage. *Biol. Bull.*, **102**: 74–89.

Dan, K., and K. Okazaki, 1956. Cyto-embryological studies of sea urchins. III. Role of the secondary mesenchyme cells in the formation of the primitive gut in sea urchin larvae. *Biol. Bull.*, **110**: 29–42.

Fox, D. L., and W. R. Coe, 1943. Biology of the California sea mussel (*Mytilus californianus*). II. Nutrition, metabolism, growth and calcium deposition. *J. Exp. Zool.*, **93**: 205–249.

Galtsoff, P. S., 1934. The biochemistry of the invertebrates of the sea. *Ecol. Monogr.*, **4**: 481–490.

Glaser, O., 1950. Note on the growth of arms in the pluteus of *Arbacia punctulata*. *Biol. Bull.*, **98**: 34–35.

Goldon, I., 1926a. The development of the calcareous test of *Echinus miliaris*. *Phil. Trans. Roy. Soc. B*, **214**: 259–312.

Goldon, I., 1926b. The development of the calcareous test of *Echinocardium cordatum*. *Phil. Trans. Roy. Soc. B*, **215**: 225–313.

Goldon, I., 1928. Skeletal development in *Arbacia*, *Echinarachinus* and *Leptasterias*. *Phil. Trans. Roy. Soc. B*, **217**: 289–334.

Herbst, C., 1904. Über die zur Entwickelung der Seeigellarven nothwendigen anorganischen Stoffe, ihre Rolle und ihre Vertretbarkeit. III. Die Rolle der nothwendigen anorganischen Stoffe. *Arch. f. Entw.*, **17**: 306–520.

Hirata, A. A., 1953. Studies on shell formation. II. A mantle-shell preparation for in vitro studies. *Biol. Bull.*, **104**: 394–397.

Hörstadius, S., 1935. Über die Determination im Verlaufe der Eiachse bei Seeigeln. *Pubbl. Staz. Zool. Napoli*, **14**: 251–429.

Jenkinson, J. W., 1911. On the development of isolated pieces of the gastrulae of the sea urchin, *Strongylocentrotus lividus*. *Arch. f. Entw.*, **32**: 269–297.

Jodrey, L. H., 1953. Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and Ca^{45}. *Biol. Bull.*, **104**: 398–407.

MacBride, E. D., 1914. The development of *Echinocardium cordatum*. *Quart. J. Micr. Sci.*, **59**: 471–486.

Mortensen, Th., 1921. Studies of the development and larval forms of echinoderms. Copenhagen.

Mortensen, Th., 1931. Contributions to the study of the development and larval forms of echinoderms. Copenhagen.

Okazaki, K., 1956. Exogastrulation induced by calcium deficiency in the sea urchin *Pseudocentrotus depressus*. *Embryologia*, **3**: 23–36.

Onoda, K., 1931. Note on the development of *Heliocidaris crassispina* with special reference to the structure of the larval body. *Memoirs College Sci. Kyoto Imp. Univ. B*, **7**: 103–134.

Orton, J. H., 1925. The conditions for calcareous metabolism in oyster and other marine animals. *Nature*, **116**: 13.

Prenant, M., 1926. Sur le déterminisme de la forme spiculaire chez les larves d'oursins. *C. R. Soc. Biol.*, **94**: 433–435.

Pouchet, C., and L. Chabry, 1889. Sur le dévelopment des larves d'oursin dans l'eau de mer privée de chaux. *C. R. Soc. Biol.*, **41**: 17–20.

Rao, K. P., and E. D. Goldberg, 1954. Utilization of dissolved calcium by a pelecypod. *J. C. C. P.*, **43**: 283–292.

Robertson, J. D., 1941. The function and metabolism of calcium in the Invertebrata. *Biol. Rev.*, **16**: 106–133.

Rulon, O., 1941. Modification of development in the sand dollar by NaSCN and Ca-free sea water. *Physiol. Zoöl.*, **14**: 303–315.

Runnström, J., 1931. Zur Entwicklungsmechanik des Skelettmusters bei dem Seeigelkeim. *Arch. f. Entw.*, **124**: 273–297.

von Ubisch, L., 1913. Die Anlage und Ausbildung des Skelettsystems einiger Echinodermen. *Zeitschr. Wiss. Zool.*, **104**: 119–156.

von Ubisch, L., 1933. Untersuchungen über Formbildung. IV. Über plutei ohne Darm, Fortsätze ohne Skelett und die Grenzen der virtuellen Keimbezirke. *Arch. f. Entw.*, **129**: 45–67.

Woodland, W., 1906. Studies in spicule formation. III. On the mode of formation of the spicular skeleton in the pluteus of *Echinus esculentus*. *Quart. J. Micr. Sci.*, **49**: 305–325.